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Induction of a balanced IgG1/IgG2 immune response to an experimental challenge with *Mycoplasma bovis* antigens following a vaccine composed of Emulsigen™, IDR peptide1002, and poly I:C

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ABSTRACT

Prevention and or control of *Mycoplasma bovis* infections in cattle have relied on the treatment of animals with antibiotics; herd management including separation and or culling infected animals; and the use of vaccines with limited protection. Due to the negative reactions and incomplete protection observed after vaccination with some bacterin-based vaccines, there is a need to put more efforts in the development of recombinant-based vaccines. However, the arsenal of antigens that may be suitable for a fully protective vaccine is rather limited at this point. We have tested a vaccine formulation containing *M. bovis* proteins formulated with adjuvants that have been shown to aid in the protection against other pathogens. After vaccinations, the animals were challenged using a BHV-1/*M. bovis* co-infection model. While the PBMC proliferation and cytokine responses to the antigens in the vaccine were negligible, humoral responses reveal that eight antigens elicit a balanced IgG1/IgG2 response although this was not enough to confer protection against *M. bovis*.

1. Introduction

Mycoplasma bovis is the causative agent of bovine chronic pneumonia and poly-arthritis syndrome (CPPS), associated with the bovine respiratory disease (BRD) complex. The presence of this pathogen remains an important economic burden to Canadian feedlot producers [1]. Considerable efforts have been put in the prevention of CPPS. Unfortunately, the increased resistance to antibiotics by some isolates combined with the antigenic plasticity of surface proteins [2,3] makes control and/or prevention of CPPS a difficult task to achieve thus the need for effective control measures in the form of vaccines. Several experimental vaccines have shown encouraging results [4–7], however field-testing of these vaccines did not have the expected outcome. A commercial vaccine for four to six weeks-old cattle (MpBGuard™, AgriLabs®) has been developed but its efficacy to protect older animals has not been tested. Recent reports describe protection in animals vaccinated with a *M. bovis* bacterin formulated with two adjuvants [8] or with attenuated *M. bovis* strains [9], however it is not clear if this latter vaccine would gain acceptance in North American markets.

Because of negative reactions observed with some *M. bovis* bacterin-based vaccines [6], identification of *M. bovis* proteins as vaccine targets remains a priority. The *M. bovis* variable surface proteins [10], the conserved P26 and P48 lipoproteins [11,12] and heat-shock proteins [13] are potential targets. We have previously identified the *M. bovis* GapC protein, membrane extracts, and protein fractions as vaccine targets however despite significant humoral responses, there was no protection as judged by the presence of lung lesions in the vaccinated animals after challenge [14,15]. Recently, we were able to detect significant humoral responses to ten *M. bovis* antigens formulated with a novel adjuvant [16]. In this work, we describe the testing of this experimental vaccine for protection against *M. bovis* in a co-infection challenge model that successfully reproduces CPPS signs observed in Canadian feedlots.

2. Materials and methods

2.1. Bacterial and virus strains

The *M. bovis* isolates Mb1 and Mb160 used in this study were described before [17,18]. These isolates carry RFLP 1 and 2 types of the gene encoding Gapdh respectively [17]. The *M. bovis* isolates were grown as described before [18] and challenge doses were

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prepared by serial dilutions of the original suspensions in the same medium and bacterial counts confirmed by plating on modified Hayflick's agar. The volume of the challenge suspension was 4 ml. The challenge strains Mb1 and Mb169 were mixed in equal proportions. The challenge dose of the strain Mb1 was 7.5 x 10^7/ml, while Mb169 was 4.9 x 10^7/ml. The BHV-1 strain 108 was grown as described before [18], aliquots were diluted in 4 ml of tissue culture medium to 1 x $10^{5.5}$ tissue culture cells infective dose 50 ($TCID_{50}$)/ml. BHV-1 was administered by nebulization and the *M. bovis* strains suspension by syringe directly into the nasal cavities.

2.2. Proteins used in the trial

The *M. bovis* membrane fractions and protein extracts together with the ten recombinant proteins used in this trial have been described elsewhere [16]. Purification of protein extracts, recombinant proteins, and *M. bovis* membranes were described before [16].

2.3. Humoral and cell-mediated immunity assays

The determination of serum antibody titres, purification of bovine peripheral-blood mononuclear cells (PBMC) and proliferation assays were performed as described before [16,19] on pre-challenge and 14 days post-*M. bovis* challenge samples. The measurement of cytokine levels in PBMC supernatants collected on the same samples was performed after incubation of PBMC with recall antigens for 24, 48 and 72h. In addition, pre- and post-challenge serum samples were collected for determination of cytokine levels. The coupling of beads and the multiplex ELISA were carried out described before [16]. The primary, and secondary antibodies together with the dilutions used are shown in supplementary Table 1.

2.4. Animal trial

All animal experiments followed the guidelines of the Care and Use of Experimental Animals provided by the Canadian Council on Animal Care and followed protocols approved by the University of Saskatchewan Animal Research Ethics Board. The trial was conducted on 16 beef calves (6–8 months old) from a cow-calf ranch in Southern Saskatchewan previously determined to have a low incidence of exposure to Bovine Herpes Virus 1 (BHV-1) and Bovine Viral Diarrhoea Virus (BVDV) as judged by negative serology. Animals with BHV-1 and BVDV titres <50 were selected. In addition, animals were determined to be *M. bovis*-free by negative cultures of nasal secretions and the average serum titre to *M. bovis* was 2500. Animals were transported to the VIDO-InterVac research farm, placed in pens, acclimatized for 10 days, received food and water "at libidum", and vaccinated as shown in supplementary Table 2. Serum titres against the *M. bovis* antigens were determined at days 0, 40 (Boost), 61 (Pre-challenge), and 77 (Post-challenge). The challenges were done as described before [18].

The animals were monitored for signs of lameness, depression and respiratory distress using the same criteria as in previous trials and euthanized as required [18]. We measured body weights and temperatures for 16 days after the viral challenges (12 days after the challenge with *M. bovis*). Necropsy of the animals consisted of gross pathologic examinations of the lungs and joints including palpation and visual observations. In addition, blood, nasal and synovial fluid samples were taken for isolation of *M. bovis*. At the end of the trial the surviving animals were euthanized and examined as above. A veterinary blinded to the vaccination status of the animals performed all clinical, clinico-pathologic and gross pathology assessments.

2.5. Recovery of M. bovis from tissue samples

Nasal secretions, lung sections from grossly normal and diseased areas, synovial fluid from stifle joints and blood samples were collected and, after enrichment by 48h in Hayflick's medium, plated for isolation of *M. bovis*. From each tissue, a random number of colonies were selected and confirmed as *M. bovis* by PCR using specific primers [17]. Genomic DNA was prepared and analysed by RFLP of the gene encoding glyceraldehyde 3-phosphate dehydrogenase, GAPDH as previously described [17,18].

2.6. Statistical analyses

We used non-parametric tests (Friedman or Kruskal-Wallis tests followed by multiple comparison tests) to compare the serum immune responses and lung lesions between the groups. Non-parametric Mann-Whitney T-tests were used to compare IgG1/IgG2 ratios and cytokine values for each incubation time of bovine PBMC obtained at pre- and post-challenge samples. All the statistic analyses were carried out using GraphPad Prism version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com. Data was considered statistically different if the *P* value was 0.05 or less.

3. Results

3.1. Serum immune responses

We previously demonstrated that vaccination with a cocktail of *M. bovis* proteins formulated with a novel adjuvant resulted in the best immune responses to the antigens and proposed that this new formulation was a good candidate for an experimental vaccine against *M. bovis* [16]. In this work, we report the results of a proof of concept trial in where animals were injected with the experimental vaccine and challenged using our combined BHV-1 – *M. bovis* challenge model.

For IgG1 and compared to group A, significant IgG1 pre-challenge responses were observed for most of the proteins used with the exception of P81 (Fig. 1); whereas only significant IgG1 post-challenge responses were detected on *M. bovis* extracts, fractions, PdhA, PepA, LppB, O256, P48, P81, and PepQ recombinant proteins (Fig. 1). For IgG2 and compared to group A, significant pre-challenge levels were observed for *M. bovis* extracts and fractions, PdhA, Tuf, PepA, LppB, O256, OppA, P48 and PepQ proteins; whereas only significant IgG2 values on the post-challenge samples were detected on PdhA, Tuf, PepA, LppB, O256, OppA, DeoB, P48, P81, and PepQ recombinant proteins (Fig. 2). For IgA and compared to group A, significant pre- challenge responses were observed for *M. bovis* extracts, PepA, LppB, P48, P81, and PepQ (Fig. 3); while significant IgA responses on post-challenge samples were detected for *M. bovis* fractions, Tuf, PepA, LppB, O256, OppA, DeoB, and P48 (Fig. 3).

In group A and compared to day 0, significant post-challenge IgG1 responses were observed for *M. bovis* extracts, Tuf, LppB, O256, OppA, DeoB, P48, P81, and PepQ. Significant IgG2 post-challenge responses were observed for *M. bovis* extracts, O256, Tuf, and PepQ, whereas there were no significant IgA responses to any of the antigens used (Suppl. Figs. S1–S3). In group B and compared to day 0, significant pre- and post-challenge IgG1 responses were observed for *M. bovis* extracts, fractions, PdhA, Tuf, PepA, LppB, O256, OppA, DeoB, P48, P81, and PepQ. Significant pre-challenge IgG2 responses were observed for *M. bovis* extracts, fractions, PdhA, Tuf, PepA, LppB, O256, P81, and PepQ; whereas significant post-challenge responses were observed for *M. bovis* extracts, fractions, PdhA, Tuf, PepA, LppB, O256, OppA, DeoB, P81,

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